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A simple and economical in-house phage technique for the rapid detection of rifampin, isoniazid, ethambutol, streptomycin, and ciprofloxacin drug resistance in *Mycobacterium tuberculosis*, directly on decontaminated sputum samples

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SUMMARY

Objectives: The early detection of drug resistance would be a boon for TB control programs. The aim of the present study was to set up a rapid phage assay for the testing of drug susceptibility of *Mycobacterium tuberculosis* to rifampin, isoniazid, ethambutol, streptomycin, and ciprofloxacin, directly on decontaminated sputum samples.
Methods: Mueller–Hinton broth was used instead of 7H9 broth to make the method more economical. Vancomycin and polymyxin B were added to the concentrated sputum samples to reduce the bacterial contamination. The phage assay on decontaminated sputum samples was compared with the proportion method using *M. tuberculosis* isolates from the same sputum samples.
Results: Phage assay results were available within 48 h for rifampin and streptomycin and within 72 h for all the other drugs. In contrast the proportion method required 4–6 weeks from the primary cultures. The sensitivity of the phage assay was in the range of 93% to 100% and specificity in the range of 96% to 100% for all the drugs tested. The interpretation of results was possible for 334 of the 370 (90.3%) acid-fast bacillus (AFB) smear-positive sputum samples by the phage assay.
Conclusions: The phage assay for the detection of drug resistance on direct decontaminated sputum samples is economical, easy to perform, and rapid.

1. Introduction

The World Health Organization (WHO) estimated that there were 9.27 million new cases of tuberculosis (TB) worldwide in 2007.[1] The countries with the highest prevalence were India (2.0 million cases), China (1.3 million), Indonesia (530 000), Nigeria (460 000), and South Africa (460 000).[1] The emergence of multidrug-resistant (MDR)-TB, and more recently, extensively drug-resistant (XDR)-TB, is a major threat to global TB control.[2–5]

MDR-TB, caused by strains resistant to at least isoniazid and rifampin, is an alarming problem for the successful management of global TB control programs, since it increases the cost of treatment and the transmission risk, and lowers the cure rate.[6] India is the number one country in terms of TB prevalence, and the prevalence rate of MDR-TB in the country is around 3% among new cases, 12% among pretreatment cases,[1] and an alarming 17% among patients who have undergone previous treatment and who have developed multidrug resistance. Three countries, namely China, India, and the Russian Federation, were found to account for 62% of the estimated global burden of MDR-TB cases.[5] MDR-TB has been reported from almost all parts of the world, primarily as a consequence of poor and irregular treatment services, resulting in increased treatment costs and also an increased risk of transmission of these resistant strains of the bacilli.[1] Also, the cure rate for MDR-TB is lower.

The early detection of MDR-TB could help in the effective management of cases and in the overall TB control program. However, current rapid phenotypic methods for assessing drug susceptibility of *M. tuberculosis*, such as BACTEC, take 2–6 weeks after laboratory isolation of the organisms. Rapid methods based on PCR are considered an alternative approach for the detection of multidrug resistance, but although there is confidence in the detection of rifampin resistance alone through the *rpo*B gene mutation,[7–13] variations in resistance genes in isoniazid are problematic. Further, the expensive set-up and a lack of expertise can be a hurdle for those developing countries with a high burden of TB.

Mycobacteriophages are used as diagnostic markers to improve and expedite the detection of viable mycobacteria in clinical samples, as well as for the determination of drug resistance from

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the primary growth. Commercial kits based on phage technology are now available for the detection of mycobacteria in clinical samples.[14–19] The phage-based assays depend on the ability of resistant mycobacteria to support phage replication after being exposed to drugs, while sensitive bacilli become inactivated and hence are not able to support phage replication. Extracellular phages are inactivated with a phagicidal agent, whereas intracellular phages are protected and replicate, causing their lysis and the release of a new phage progeny detected by the production of plaques on a fast-growing *Mycobacterium smegmatis* (indicator strain) lawn.

There are a few reports of phage-based assays carried out on direct decontaminated sputum samples for the detection of rifampin resistance, with fewer for isoniazid resistance. The detection of multidrug resistance requires the determination of resistance to both isoniazid and rifampin. Further, phage-based assays for streptomycin, ethambutol, and fluoroquinolones have not been reported for direct sputum samples.

The purpose of this study was to evaluate an in-house and standardized phage amplification method using direct decontaminated sputum samples for the determination of rifampin, isoniazid, ethambutol, streptomycin, and ciprofloxacin drug resistance, and to compare the results to those acquired using the gold standard proportion method.

2. Materials and methods

2.1. Population

Three hundred and seventy cases of acid-fast bacillus (AFB) smear-positive pulmonary TB were referred to the Microbiology Laboratory of Choithram Hospital and Research Centre (CHRC), Indore, India during the period January 2008 to August 2010. Of these cases, 62% were on anti-TB treatment and 40% had received irregular treatment for more than 2 years and showed an inadequate therapeutic response. The proportion of males was 65% and cases were aged in the range of 18–65 years.

The study was approved by the Research and Ethics Committee of CHRC, Indore, India. The Ethics Committee has guidelines based on the Declaration of Helsinki.

2.2. Samples

Sputum samples were collected in wide-mouth sterile containers. AFB smear by auramine O fluorescent method was carried out on the samples.[20] Prior to concentration, sputum samples were checked for mycobacteria, and all 370 smear-positive samples were included in the study. The density of AFB in the smear was recorded as per WHO guidelines.[21] Of the sputum samples included in the study, 52 were recorded as 1–3 AFB/100 high-power fields, 122 samples as 1+, 113 samples as 2+, and 83 samples as 3+.

The *N*-acetyl-L-cysteine–alkali digestion method, as described by Kubica et al.,[22] was used for the concentration of sputum samples. After concentration, 5 units/ml polymyxin B (Samarth Pharmaceutical Ltd, India) and 2 μg/ml vancomycin (Sigma, USA) were added to prevent bacterial contamination.

2.3. Phage assay protocol

The phage assay protocol was developed for isoniazid, rifampin, ethambutol, streptomycin, and ciprofloxacin drug susceptibility testing directly on decontaminated sputum samples from pulmonary TB cases.

All drugs were from Sigma-Aldrich Chemicals GmbH, Steinheim, Germany. Isoniazid, ethambutol, and streptomycin stock solutions at 10 mg/10 ml were made in distilled water and filtered through a 0.2-μ pore size membrane. Rifampin and ciprofloxacin stock solutions (10 mg/10 ml) were made in dimethylformamide (Sigma, USA). All the stock drug solutions were aliquoted and preserved at −70 °C until use. The working solution was prepared in Mueller–Hinton broth (BD Difco, USA; Cat. No. 275730) and had no supplemented cation. The final drug concentrations used were: isoniazid 0.4 μg/ml, rifampin 2 μg/ml, ethambutol 16 μg/ml, streptomycin 2 μg/ml, and ciprofloxacin 4 μg/ml. Plain Mueller–Hinton broth without drug served as the control plate. Mueller–Hinton agar (BD Difco, USA) supplemented with 0.4% glycerol and 1% glucose was used for plate pouring in the phage assay.

M. smegmatis mc^2155 strain was used for the propagation of mycobacteriophages on Mueller–Hinton agar. Mycobacteriophage (D29) was propagated on *M. smegmatis*, as described by Sarkis and Hatfull.[23] The supernatant containing phages was passed through a 0.22-μ millipore filter membrane and stored at −20 °C in a deep freeze. The phage titer was determined using a standard spot test.

With regards to the drug exposure time period for *M. tuberculosis* strains, decontaminated and concentrated sputum samples were exposed to rifampin and streptomycin for 24 h, while the exposure time for the other drugs was 48 h; this was determined on the basis of our preliminary work and on other studies.[24–34]

In brief, the assay was carried out in 10-ml plastic screw-capped tubes (Nunc, USA). Five hundred microliters of decontaminated sputum specimen was mixed with 500 μl of the drug. A wild-type laboratory isolate drug-resistant strain and *M. tuberculosis* $H_{37}Ra$ were included in all batches. Plain Mueller–Hinton broth without drug served as the 0 μg/ml concentration of the drug. The tubes were incubated for 24/48 h at 37 °C.

Two hundred microliters of mycobacteriophage D29 (10^8 pfu/ml) were added to the respective tubes and incubated for 90 min at 37 °C. The extracellular phages were inactivated with 200 μl of phagicidal agent ferrous ammonium sulfate (FAS; 30 mM) with a contact time of 10 min. Next, the contents of the tubes were transferred to tubes containing 10 ml Mueller–Hinton broth (18 × 150 mm tubes) and the tubes vortexed to dilute the effect of the FAS. Separate OADC (oleate–albumin–dextrose–catalase) or citrate was not added as a neutralizing agent for FAS. One milliliter of the broth mixture and 1 ml of *M. smegmatis* suspension was dispensed into a sterile disposable Petri dish (90 mm); 10 ml of molten Mueller–Hinton agar supplemented with glycerol and glucose was added to the Petri dish and mixed by rotating clockwise and anticlockwise. The plates were left for 10 min for solidification, transferred to an incubator at 37 °C, and left overnight. Results were analyzed by counting the plaques. A growth control containing no drug was included for each test sample. The negative control plate had to have more than 100 plaques. If the plaque count was less, the experiment was repeated with double the volume (1 ml) of decontaminated sputum sample. The strains were considered resistant if the reduction in plaque count was less than 80% for the drug compared to the 'no drug' control plate.

2.4. Drug susceptibility testing by proportion method

The proportion method was carried out on Lowenstein–Jensen medium.[35] The recommended critical concentrations of isoniazid (0.2 μg/ml), rifampin (40 μg/ml), ethambutol (2 μg/ml), streptomycin (4 μg/ml), and ciprofloxacin (2 μg/ml) were used.

2.5. Statistical analysis

Data were analyzed using Bayes Theorem and 2 × 2 contingency tables to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratios

Table 1
The phage assay and its comparison with the proportion method for the detection of drug resistance to isoniazid, rifampin, streptomycin, ethambutol, and ciprofloxacin ($N = 334$ sputum AFB smear-positive samples from pulmonary TB cases)

Drug	Resistant by proportion method	Concordant: Resistant by phage assay	Discordant: Resistant by proportion method but susceptible by phage assay	Susceptible by proportion method	Concordant: Susceptible by phage assay	Discordant: Susceptible by proportion method but resistant by phage assay	Sensitivity (%)	Specificity (%)	PPV	NPV	LR+	LR−
INH	197 (59%)	196	1	137 (41%)	136	1	98	98	98	98	42	0.017
RIF	143 (43%)	143	0	191 (57%)	191	0	100	100	100	100	Infinity	0.0
STR	183 (55%)	182	1	151 (45%)	151	0	98	100	100	98	Infinity	0.017
EMB	106 (32%)	105	1	228 (68%)	226	2	97	97	94	99	34	0.031
CIP	93 (28%)	91	2	241 (72%)	238	3	93	96	90	97	23	0.069

AFB, acid-fast bacillus; TB, tuberculosis; INH, isoniazid; RIF, rifampin; STR, streptomycin; EMB, ethambutol; CIP, ciprofloxacin; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR−, negative likelihood ratio.

(LR), using StatsDirect statistical software version 2.7.2 (StatsDirect, UK). The level of agreement between the phage assay and the proportion method for the detection of drug resistance in *M. tuberculosis* was measured using the kappa index (κ) of agreement.

3. Results

Three hundred and seventy AFB-positive sputum samples were selected for the phage assay. The density of AFB in the sputum samples was 1–3 AFB/100 high-power fields in 52 samples, 1+ in 122 samples, 2+ in 113 samples, and 3+ in 83 samples.

The results of the phage assay and its comparison to the proportion method are shown in Table 1.

After decontamination, the sputum samples were subjected to the phage assay and culture. The phage assay was carried out on the direct decontaminated sputum samples, whereas the proportion method was carried out on the culture isolated from the same sputum sample.

By the proportion method on the mycobacterial isolates, mycobacterial resistance to isoniazid was found in 197 (58%), to rifampin in 143 (43%), to streptomycin in 183 (55%), to ethambutol in 106 (32%), and to ciprofloxacin in 93 (28%). The 143 mycobacterial isolates that were resistant to rifampin were also resistant to isoniazid. In addition 54 samples showed resistance to isoniazid but were sensitive to rifampin.

Concordance of the phage assay was 99.5% for isoniazid, 100% for rifampin, 99.4% for streptomycin, 99.4% for ethambutol, and 97.8% for ciprofloxacin. Discordance was seen for two, nil, one, three, and five samples in the case of isoniazid, rifampin, streptomycin, ethambutol, and ciprofloxacin, respectively.

The sensitivity of the phage assay for isoniazid was 98%, for rifampin was 100%, for streptomycin was 98%, for ethambutol was 97%, and for ciprofloxacin was 93% (Table 1). The specificity, positive predictive value, and likelihood ratios are given in Table 1.

The level of agreement between the phage assay and the proportion method was measured using Cohen's kappa index (κ) of agreement: for isoniazid this was 0.9877 (standard error (SE) 0.0546), for rifampin 1.000 (SE 0.0547), for streptomycin 0.994 (SE 0.0546), for ethambutol 0.9795 (SE 0.0545), and for ciprofloxacin 0.9636 (SE 0.0543).

Seventeen of the 370 AFB smear-positive samples did not yield growth on Lowenstein–Jensen medium and failed to give plaques in the phage assay. Thirteen of the 370 sputum samples showed bacterial/fungal contamination on the phage assay plates and did not give interpretable results. Seventeen samples had fewer than 100 plaques on the phage assay plates. Hence, the experiment was repeated using 1 ml volume of decontaminated sample, and interpretable results were obtained in 11 cases. The plaque count was persistently less than 100 on the negative control plates for six samples and hence these could not be used for comparison.

Interpretable results were obtained by the proportion method for 95.4% of the samples, while the phage assay offered results for 90.3% of the samples. However, it should be mentioned that the results were available within 48–72 h for the phage assay on the direct samples, while the proportion method took 2–6 weeks after the primary isolation.

4. Discussion

The conventional proportion method takes 4–6 weeks after the isolation of *M. tuberculosis*. The PCR-based method offers results within 8 h, however the set-up requires expensive equipment and skilled personnel. Attempts are now being made by international agencies to make the Xpert MTB/RIF test available in developing countries.[36] Further, molecular methodologies have gained credence only for the detection of rifampin resistance.[7–13] A phage-based assay was developed by Wilson et al. and was applied to rifampin and isoniazid susceptibility testing in clinical isolates of *M. tuberculosis*.[37] Commercial phage-based assays are available only for the detection of resistance to rifampin.[14,38,39]

A number of research papers have documented the utility of the technique for the determination of drug resistance in mycobacterial isolates.[24–34] The approach was used by Albert et al.[40] for the detection of rifampin resistance in *M. tuberculosis* cells directly from sputum samples.

Today the increase in multiple drug resistance appears to be a worldwide problem, limiting the success of TB control programs, and is one of the major challenges for the directly observed therapy (DOT)-plus program. A recently updated meta-analysis describes five publications on the detection of rifampin resistance on direct decontaminated sputum samples.[41] However, there are no published data to support the use of phage assays for the detection of isoniazid, ethambutol, streptomycin, and ciprofloxacin resistance on direct clinical samples. Hence, the aim of the present study was to standardize an in-house phage assay to determine resistance to all of the first-line anti-TB drugs (isoniazid, rifampin, streptomycin, and ethambutol), and also to fluoroquinolones, directly on decontaminated sputum samples. Fluoroquinolones are widely used along with other anti-TB drugs in India and therefore were also included in the present study.

Middlebrook 7H9 medium was replaced with Mueller–Hinton broth to make the assay economical. The cost of the commercial phage-based assay (FASTPlaque TB-RIF, Biotec Laboratories Ltd, UK) for rifampin resistance is approximately US$ 10 per test, while the cost of consumables for the in-house phage-based assay is less than US$ 1 per sample.

Seventeen of the 370 sputum samples failed to yield plaques in the phage assay and also did not show growth on Lowenstein–Jensen medium, indicating a lack of viable mycobacteria in the samples. For seven of the samples (not shown in the results), the

density analysis showed only occasional AFB in the smear, and the other 10 cases were on anti-TB drugs. The other limitation for a few of the samples was the problem of contamination. Mole et al. reduced the possibility of bacterial contamination from 14.3% to 0.8% by the use of nystatin, oxacillin, and aztreonam.[42] In the present study, a combination of polymyxin B and vancomycin was used due to the high prevalence of methicillin-resistant *Staphylococcus aureus* and extended-spectrum beta-lactamase producers in the local setting, but still 13 of 370 samples (3.5%) failed to give interpretable results on account of bacterial contamination.

To overcome the problem of a low plaque count in the 17 samples, the samples were reprocessed using 1 ml instead of 0.5 ml of decontaminated sputum sample, and 11 of them then gave interpretable results. However, six still yielded low plaque counts. The observation suggests phage inhibitory effects of substances present in the sputum samples, as mentioned by Albert et al.[40] and Muzaffar et al.[17] The possibility of a low density of viable mycobacteria in the sputum samples also needs to be considered.

In the present study contaminated results were seen in 13 and indeterminate results in six, i.e., only 19/370 (5.1%) specimens. The studies by Butt et al.,[34] Albert et al.,[40] and Mole et al.,[42] reported 17–36% samples as contaminated/indeterminate by the phage assay. The improved performance in the present study appears to be due to the inclusion of vancomycin and polymyxin and the performance of the in-house assay at our end. It should be mentioned that the other studies reported only rifampin resistance and did not include other drugs (isoniazid, streptomycin, ethambutol, and ciprofloxacin).

The overall results based on sensitivity, specificity, negative and positive predictive values, likelihood ratios (LR), and agreement with the comparator based on the kappa value are good in the present study (Table 1). The results were interpretable for 334/370 (90.3%) of the samples and were available in 48–72 h against an average period of 4–6 weeks of primary culture by the proportion method. The cost per test for isoniazid, rifampin, streptomycin, ethambutol, and ciprofloxacin sensitivity testing by the in-house phage assay was less than US$ 1. The commercially available FASTPlaque method costs approximately US$ 10 per test and has been standardized only for the detection of rifampin resistance for decontaminated sputum samples.[40] The use of Mueller–Hinton broth and agar in the present study is far more economical than the 7H11 used in the FASTPlaque assay.

The threshold for defining susceptibility was more than 80% for all the drugs in the present study. However, a more than 99% reduction in plaque count was observed for rifampin and streptomycin, and this appeared to be due to high and rapid bactericidal activity of the drugs for mycobacteria. This observation is consistent with the data from another mycobacteriophage-based assay, the luciferase-reporter phage (LRP) assay, in which a 99% reduction in the light signal was seen after an overnight incubation of susceptible isolates with streptomycin.[43] These two drugs required a shorter time exposure – 24 h – compared to the other drugs, which required 48 h exposure in the present method, and the reason appears to be the rapid bactericidal activity of rifampin and streptomycin. Similar exposure times were observed in another study.[24] The mode of action of the drug may also be a contributory factor, e.g., activity at the transcription/translation level (streptomycin, rifampin) and at the cell wall level (e.g., isoniazid, ethambutol), as suggested by Jacobs et al.[44]

In the case of streptomycin and rifampin, the drug concentration required for exposure in the phage assay was less than the recommended concentration for the proportion method and this also appears to be due to the rapid bactericidal activity of the drugs. The concentrations of the other drugs used in the study were higher than recommended for the proportion method, possibly because of slower drug activity. In the proportion method a lower concentration may suffice since the exposure is over a long period.

The phage assay offers a simple and rapid testing method for the detection of mycobacterial resistance using direct clinical specimens like sputum, and could be a boon for the DOT-Plus program in economically developing countries where the burden of TB is very high. The methodology does not demand expensive equipment. Our laboratory is presently working to standardize the phage assay for other anti-TB drugs and the preliminary results are very encouraging. The rapidity of the results and the cost-effectiveness of the method is expected to offer great help in reducing the transmission of drug-resistant TB infections and in the effective management of MDR-TB cases.

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References

1. World Health Organization. Global tuberculosis control: epidemiology, strategy, financing: WHO report 2009. WHO/HTM/TB/2009.411. Geneva: World Health Organization; 2009.
2. Matteelli A, Migliori GB, Cirillo DM, Centis R, Girardi E, Raviglione MC. Multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis*: epidemiology and control. *Expert Rev Anti Infect Ther* 2007;**5**:857–71.
3. Migliori GB, Loddenkemper R, Blasi F, Raviglione MC. 125 years after Robert Koch's discovery of the tubercle bacillus: the new XDR-TB threat. Is "science" enough to tackle the epidemic? *Eur Respir J* 2007;**29**:423–7.
4. Shah NS, Wright A, Bai GH, Barrera L, Boulah Bal F, Martin-Casabona N, et al. Worldwide emergence of extensively drug-resistant tuberculosis. *Emerg Infect Dis* 2007;**13**:380–7.
5. Zignol M, Hosseini MS, Wright A, Weezenbeek CL, Nunn P, Watt CJ, et al. Global incidence of multidrug-resistant tuberculosis. *J Infect Dis* 2006;**194**:479–85.
6. Yew WW, Chau CH. Drug-resistant tuberculosis in the 1990s. *Eur Respir J* 1995;**8**:1184–92.
7. Garcia L, Alonso-Sanz M, Rebollo MJ, Tercero JC, Chaves F. Mutations in the *rpo*B gene of rifampin-resistant *Mycobacterium tuberculosis* isolates in Spain and their rapid detection by PCR–enzyme-linked immunosorbent assay. *J Clin Microbiol* 2001;**39**:1813–8.
8. Hillemann D, Rüsch-Gerdes S, Richter E. Application of the genotype MTBDR assay directly on sputum specimens. *Int J Tuberc Lung Dis* 2006;**10**:1057–9.
9. Hillemann D, Rüsch-Gerdes S, Richter E. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. *J Clin Microbiol* 2007;**45**:2635–40.
10. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, et al. Detection of rifampin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993;**341**:647–50.
11. Watterson SA, Wilson SM, Yates MD, Drobbiewski F. Comparison of three molecular assays for rapid detection of rifampin resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* 1998;**36**:1969–73.
12. Williams DL, Spring L, Collins L, Miller LP, Heifets LB, Gangadharam PR, et al. Contribution of *rpo*B mutations to development of rifamycin cross-resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1998;**42**:1853–7.
13. Williams DL, Spring L, Salfinger M, Gillis TP, Persing DH. Evaluation of polymerase chain reaction-based universal heteroduplex generator assay for direct detection of rifampin susceptibility of *Mycobacterium tuberculosis* from sputum specimens. *Clin Infect Dis* 1998;**26**:446–50.
14. Albert H, Heydenrych A, Brookes R, Mole RJ, Harley B, Subotsky E, et al. Performance of a rapid phage-based test, FASTPlaqueTB to diagnose pulmonary tuberculosis from sputum specimens in South Africa. *Int J Tuberc Lung Dis* 2002;**6**:529–37.
15. Alcaide F, Gali N, Dominguez J, Berlanga P, Blanco S, Orus P, et al. Usefulness of a new mycobacteriophage-based technique for rapid diagnosis of pulmonary tuberculosis. *J Clin Microbiol* 2003;**41**:2867–71.
16. Barman P, Gadre D. A study of phage based diagnostic technique for tuberculosis. *Indian J Tuberc* 2007;**54**:36–40.
17. Muzaffar R, Batool S, Aziz F, Naqvi A, Rizvi A. Evaluation of the FASTPlaqueTB assay for direct detection of *Mycobacterium tuberculosis* in sputum specimens in Pakistan. *Int J Tuberc Lung Dis* 2002;**6**:635–40.
18. Park DJ, Drobniewski FA, Meyer A, Wilson SM. Use of a phage-based assay for phenotypic detection of mycobacteria directly from sputum. *J Clin Microbiol* 2003;**41**:680–8.

19. Trollip A, Albert H, Maskell T. Bacteriophage-based technologies for the rapid diagnosis and drug susceptibility testing of tuberculosis. *Am Clin Lab* 2001;**20**:39–42.
20. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Washington Jr CM. Color atlas and textbook of diagnostic microbiology, 5th ed., Philadelphia: JB Lippincott; 1997.
21. World Health Organization. Laboratory services in tuberculosis control. Part II: microscopy. WHO/TB/98.258. Geneva: WHO; 1998.
22. Kubica GP, Dye WE, Cohn ML, Middlebrook G. Sputum digestion and decontamination with *N*-acetyl-L-cysteine sodium hydroxide for culture of mycobacteria. *Am Rev Respir Dis* 1963;**87**:775–9.
23. Sarkis JS, Hatfull GF. Mycobacteriophages. *Methods Mol Biol* 1998;**101**:145–73.
24. Eltringham IJ, Wilson SM, Drobniewski FA. Evaluation of a bacteriophage-based assay (phage amplified biologically assay) as a rapid screen for resistance to isoniazid, ethambutol, streptomycin, pyrazinamide, and ciprofloxacin among clinical isolates of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1999;**37**:3528–32.
25. Galí N, Domínguez J, Blanco S, Prat C, Alcaide F, Coll P, et al. Use of a mycobacteriophage-based assay for rapid assessment of susceptibilities of *Mycobacterium tuberculosis* isolates to isoniazid and influence of resistance level on assay performance. *J Clin Microbiol* 2006;**44**:201–5.
26. Galí N, Domínguez J, Blanco S, Prat C, Quesada MD, Matas L, et al. Utility of an in-house mycobacteriophage-based assay for rapid detection of rifampin resistance in *Mycobacterium tuberculosis* clinical isolates. *J Clin Microbiol* 2003;**41**:2647–9.
27. Yzquierdo SL, Lemus D, Echemendia M, Montoro E, McNerney R, Martin A, et al. Evaluation of phage assay for rapid phenotypic detection of rifampicin resistance in *Mycobacterium tuberculosis*. *Ann Clin Microbiol Antimicrob* 2006;**5**:11.
28. Chauca JA, Palomino JC, Guerra H. Evaluation of rifampicin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* by a mycobacteriophage D29-based assay. *J Med Microbiol* 2007;**56**:360–4.
29. Traore H, Ogwang S, Mallard K, Joloba ML, Mumbowa F, Narayan K, et al. Low-cost rapid detection of rifampicin resistant tuberculosis using bacteriophage in Kampala, Uganda. *Ann Clin Microbiol Antimicrob* 2007;**6**:1.
30. da Silva PA, Boffo MM, de Mattos IG, Silva AB, Palomino JC, Martin A, et al. Comparison of redox and D29 phage methods for detection of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*. *Clin Microbiol Infect* 2006;**12**:293–6.
31. Eltringham IJ, Drobniewski FA, Mangan JA, Butcher PD, Wilson SM. Evaluation of reverse transcription-PCR and a bacteriophage-based assay for rapid phenotypic detection of rifampin resistance in clinical isolates of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1999;**37**:3524–7.
32. McNerney R, Mallard K, Urassa HM, Lemma E, Donoghue HD. Colorimetric phage-based assay for detection of rifampin-resistant *Mycobacterium tuberculosis*. *J Clin Microbiol* 2007;**45**:1330–2.
33. McNerney R, Kiepiela P, Bishop K, Nye PM, Stoker NG. Rapid screening of *Mycobacterium tuberculosis* for susceptibility to rifampicin and streptomycin. *Int J Tuberc Lung Dis* 2000;**4**:69–75.
34. Butt T, Ahmad RN, Afzal RK, Mahmood A, Anwar M. Rapid detection of rifampicin susceptibility of *Mycobacterium tuberculosis* in sputum specimens by mycobacteriophage assay. *J Pak Med Assoc* 2004;**54**:379–82.
35. Canetti G, Froman F, Grosset J, Hauduroy P, Langerova M, Mahler HT, et al. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bull World Health Organ* 1963;**29**:565–78.
36. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid Molecular detection of tuberculosis and rifampin. *N Engl J Med* 2010;**363**:1005–15.
37. Wilson SM, Al-Suwaidi Z, McNerney R, Porter J, Drobniewski FA. Evaluation of a new rapid bacteriophage-based method for the drug susceptibility testing of *Mycobacterium tuberculosis*. *Nat Med* 1997;**3**:465–8.
38. Albert H, Heydenrych A, Mole R, Trollip A, Blumberg L. Evaluation of FASTPlaqueTB-RIF, a rapid, manual test for the determination of rifampicin resistance from *Mycobacterium tuberculosis* cultures. *Int J Tuberc Lung Dis* 2001;**5**:906–11.
39. Albert H, Trollip AP, Mole RJ, Hatch SJ, Blumberg L. Rapid indication of multi-drug-resistant tuberculosis from liquid cultures using FASTPlaqueTB-RIF, a manual phage-based test. *Int J Tuberc Lung Dis* 2002;**6**:523–8.
40. Albert H, Trollip A, Seaman T, Mole RJ. Simple, phage-based (FASTPlaque) technology to determine rifampicin resistance of *Mycobacterium tuberculosis* directly from sputum. *Int J Tuberc Lung Dis* 2004;**8**:1114–9.
41. Minion J, Pai M. Bacteriophage assays for rifampicin resistance detection in *Mycobacterium tuberculosis*: updated meta-analysis. *Int J Tuberc Lung Dis* 2010;**14**:941–51.
42. Mole R, Trollip A, Abrahams C, Bosman M, Albert H. Improved contamination control for a rapid phage based rifampicin resistance test for *Mycobacterium tuberculosis*. *J Med Microbiol* 2007;**56**:1334–9.
43. Riska PF, Jacobs Jr WR. The use of luciferase-reporter phage for antibiotic-susceptibility testing in mycobacteria. *Methods Microbiol* 1998;**101**:431–55.
44. Jacobs Jr WR, Barletta RG, Udani R, Chan J, Kalkut G, Sosne G, et al. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* 1993;**260**:819–22.